Remarks

Claims

Claims 11, 14, 57-60 and 69-84 are currently pending in the application. Claims 1-10, 12-13, 15-27, and 61-68 are cancelled. Claims 28-56 are withdrawn. As a courtesy, claims 11, 14, 57-60 and 69-84 are presented in clean form as an appendix attached herewith immediately following the response, in case the Examiner, like the Applicants, finds the revised claim format heavy on information and light on clarity of the actual claims to be examined.

Claims 11 and 57 have been amended to encompass a "screening to identify" subpart of b) that comprises the sub-steps of "screening to identify a nucleic acid composition homologous to portions of the endogenous gene that mutes at the level of post-transcription." Support for this amendment is found in the specification on p. 13, lines 13-23; p. 32, lines 22-26-16; p. 33, lines 24-28; and Examples 9 and 10, pp. 25-28 wherein several sequences homologous to various regions of the endogenous target gene pro- α1(I)collagen [see p. 25, lines 7-8 and p. 25, line 28 through p. 26, line10 wherein constructs containing either 3521, 2296, 947 or 22bp of the promoter plus 115 bp of the untranslated portion of exon-1 of the endogenous gene were tested for muting effects, and p. 27, line 9 through p. 28, line 5 wherein constructs containing -222 to +115 of intron 1, -222 to + 585, and -222 to the end of exon 5 were investigated for muting effects]. In addition, Example 15 on p. 32, lines 18-24 describes how to isolate and test – i.e. screen – for fragments of the HIV *tat* gene have muting activity.

Claims 11, 57, and 69 have also been amended in part c) from "muting expression of the exogenous gene *wherein muting comprising muting at the level of post-*

transcription in the population as a whole" such that the muting limitation in part c) was moved, in a slightly different form, to the identifying of part b) ii) such that the method requires "identifying a first sequence of muting nucleic acid composition .... *that mutes at the level of post-transcription*." Support for this re-structuring of these claims is as before for the original post-transcriptional muting limitation, namely, p. 14, lines 24-27; p. 23, lines 3-4 and also is found on p. 30, lines 15-18. Applicants respectfully submit that the amended form of the claim does not change the substantive requirement for the muting sequence to effect post-transcriptional muting and does not represent addition of new matter. In the present format, the post-transcriptional muting limitation is now tied directly to the muting nucleic acid composition, rather than present as a nebulous conclusive effect after delivery of the muting nucleic acid composition.

Finally, claims 11, 57 and 69 have been amended in part c) to state that "muting is independent of integration *or level of* expression..." rather than independent of integration, expression or transcription. Support for this amendment is found as before in the application on p. 13, lines 6-12 and 21-24; p. 14, lines 7-8; p. 21, lines 22-23; p. 24, lines 4-10; and Example 12, particularly p. 29, lines 4-5 and Example 14, p. 30 line 25 through p. 31, line 9.

Claim 14 has been amended to ensure a proper antecedent basis (first sequence of muting nucleic acid composition) relative to the currently amended independent claim (11) from which it depends.

Claim 60 has been amended to use proper English relative to noun/verb agreement (the cultured population ... is a population of rodent cells).

New claims 70-84 have also been added. Support for these new claims is as follows:

Support for new claims 70, 71, 77 and 81 is found as above in the application on p. 13, lines 13-24; p. 32, lines 22-26-16; p. 33, lines 24-28; and Examples 9 and 10, pp. 25-28 wherein several sequences homologous to various regions of the endogenous target gene pro- α1(I)collagen [see p. 25, lines 7-8 and p. 25, line 28 through p. 26, line10 wherein constructs containing either 3521, 2296, 947 or 22bp of the promoter plus 115 bp of the untranslated portion of exon-1 of the endogenous gene were tested for muting effects, and p. 27, line 9 through p. 28, line 5 wherein constructs containing -222 to +115 of intron 1, -222 to + 585, and -222 to the end of exon 5 were investigated for muting effects].

Further support, relating to the limitation of muting at the level of transcription, is found in the application on p. 14, lines 19-22; p. 27, lines 15-25; p. 28, lines 1-3; and p. 30, lines 9-13. Support relating to muting at the level of transcription by certain sequences combined with muting at the level of post-transcription by other muting sequences is found on p. 14, lines 24-27; p. 20, lines 27-29; p. 23, lines 4-8; p. 26, lines 22-28; and p. 30, lines 3-6 and 13-18. Support for a single nucleic acid composition comprising a muting sequence that mutes at the level of post-transcription and a muting sequence that mutes at the level of transcription is found in the specification on p. 14, lines 19-27; Example 7, p. 22, lines 17-20 and p. 23, lines 3-4; p. 26, lines 20 through p. 27, lines 20-28; p. 30, lines 15-20

Support for new claims 72-76 is found in the application on p. 4, lines 7 and lines 11-13 and original claims 17 and 24 (claims 72-74); and p. 3, lines 18-19 and original claim 13 (claim 76).

Support for new claims 78-79 is found in the application on p. 3, lines 26-29; p. 15, lines 11-13; in original claim 11; and in Examples 2, 6, 7, and 9-13.

Support for new claims 80-84 is found in the application as above for claims 57 and 69, and additionally in the specification on p. 10, lines 9-16; p. 13, lines 13-24; Example 2, pp. 16-18, and Examples 6 and 9-13.

Rejections Under 35 U.S.C. § 112, paragraph one

The Examiner has maintained the rejections of claims 11, 13-18, 22-24, and 57-67 because, as stated previously, ".... the specification, while being enabling for a method of muting expression of a α1(I) procollagen in cultured rodent fibroblasts, ... does not reasonably provide enablement for the claimed method comprising other embodiments." See Office Action, p. 3, main paragraph. The Examiner goes on to say that "a single working example directed to muting of a single gene, α1(I) procollagen gene, cannot be extrapolated to embrace muting of all genes as claimed." The Examiner reiterates the position that "there is no teaching .... that would suggest whether the muting effect is general." See Office Action, p.4 – second paragraph.

Applicants respectfully submit that there is no requirement for more than a single working example, as set forth in MPEP § 2164.02, which states that an applicant need not describe all actual embodiments. Prophetic examples of how one might perform other embodiments of the invention are proper, and in the present case, Example 15 is a

description of prophetic examples which explain how one might screen for muting nucleic acids for other genes. In addition, as explained in the last response submitted February 13, 2003, the mechanistic evidence provided in the specification showing that muting occurs at both the level of transcription and post-transcription (see application, pp. 14, lines 19-27; 23, lines 3-4; and 30, lines 15-20) provides evidence to one skilled in the art that the muting effect is a general effect.

As explained in the February 13, 2003 response and the telephone interview of August 4, 2003, Applicants are the first to show gene muting in any system and more significantly, in an animal system. Although co-suppression has been shown in plants, *Drosophila*, fungi and *C. elegans*, co-suppression or any type of gene silencing, prior to the Applicants' publication in 1999 and subsequent filing of this application, had never been shown in higher animals.

Applicants respectfully submit that according to MPEP § 2164.04, the burden is on the Examiner to prove a *prima facie* case of lack of enablement, and relative to a single working example, the Examiner must evaluate all the facts and evidence and state why one would not be able to extrapolate the one example (in this case one working and several prophetic) to the entire scope of the claims. Applicants respectfully submit that the Examiner has not proven the *prima facie* case, and has not sufficiently stated why one would not be able to extrapolate the working example plus prophetic examples provided, to the entire scope of the claims.

Even assuming, *arguendo*, that the Examiner has made a *prima facie* case, Applicants rebut the arguments provided by the Examiner and respectfully submit that those skilled in the art immediately understood, upon publication of Applicants' 1999

Molecular and Cellular Biology article (the basis for this application) the implications of the findings in rodent cells relative to all animal systems (including *in vivo* animal systems). Applicants' breakthrough results are repeatedly cited by others, both in journal articles and patent applications (e.g. U.S. 20020108142) relating to gene silencing in animal systems, all later than the publication date of Applicants' 1999 publication in Molecular and Cellular Biology. One example of a journal article is the Nature publication titled "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells" by Tuschl et al. (a leading researcher in the field of RNAi) published in 2001 (vol. 411, pp. 494-498) and provided herewith as a supplement to the response. In the Nature article, the introductory paragraph states that given the results seen by Applicants in their 1999 publication with rat fibroblasts, the "apparent lack of RNAi in mammalian cell cultures was unexpected." Consequently, Tuschl (and presumably others) raced to prove that RNAi must also be present in common mammalian systems. (Others had already, subsequent to Applicants' publication, shown RNAi existed in mouse oocytes and early embryos – see references 13 and 14 of the Tuschl et al. Nature article).

Thus, Tuschl et al. report in the Nature article, for the first time, siRNA-mediated gene silencing in mammalian cells wherein the expression of several genes in two human cell lines were greatly reduced, indicating the general applicability of RNAi to a broad spectrum of genes and systems. Whereas RNAi had previously been limited to lower animal forms and plants, Applicants' seminal 1999 publication showing muting with an exogenous gene in a mammalian cell system immediately ignited the RNAi world to explore mammalian systems, because Applicants' publication indicated to anyone skilled

in the art that the muting effect seen by Applicants must be going through small dsRNA molecules – which in fact turned out to be the case, as shown by Tuschl et al. and subsequently many others.

One of the inventors, Dr. Bahramian, has also placed this technology in perspective relative to the applicable art in the field, both at the time the application was filed and immediately following publication of the muting phenomena in the 1999 Molecular and Cellular Biology journal, as detailed in the accompanying 37 CFR §1.132 Declaration ("Bahramian Declaration"), attached herewith. As pointed out by Dr. Bahramian, it was known before the filing date of this application that "...dsRNA >30 bp in the cytoplasm of mammalian cells can trigger profound physiological reactions" and lead to an interferon response (see Declaration, p. 3, para. 4, last sentence). Dr. Bahramian also asserts that the 1999 paper of which he is a co-author was the first report of transgene-induced gene silencing in a mammalian system, and the paper linked the specific mRNA degradation in mammalian cells observed with in vivo production of short dsRNA that act as intermediary molecules in the muting effect observed (see Declaration, p. 3, para. 5).

And, as asserted previously, Dr. Bahramian notes that almost immediately after publication of the 1999 Molecular and Cellular Biology paper, other researchers proved that small dsRNA sequences, "small interfering RNAs – siRNAs- were the mediators of sequence-specific mRNA degradation in the muting observed in mammalian cells (see Declaration, p. 3, para. 6).

Therefore, Applicants respectfully submit that not only is the muting effect generally applicable to other mammalian genes and cell systems, including human cell

systems and genes, the muting effect is applicable to rodent *in vivo* systems since later researchers (i.e. in 2000) have shown RNAi to occur in mouse oocytes and early embryos. Relative to the phenotypic change expected, a decrease in a rodent model that overproduces collagen, for example a rodent model with a sclerotic liver, could be readily followed by reduction in the amount and severity of sclerosis. Similarly, a decrease in a rodent model expressing normal levels of collagen could be monitored using both PCR analysis of collagen mRNA expression and/ or combined observation of collagen containing tissue, such as appearance of skin, strength of joints, etc.

As set forth in MPEP § 2164.03, the burden is on the Examiner to show lack of correlation between an *in vitro* animal model and an *in vivo* application. "If the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the Examiner has evidence that the model does not correlate." For a review of the current state of RNAi in various cell systems, Applicants also have provided herewith a copy of a 2002 article in Science, detailing that dsRNA can now be used in a wide variety of eukaryotes to suppress the expression of virtually any gene See Appendix B, Zamore, P., *Science*, 296, 1265 (2002). Given that shortly after this application was filed RNAi was shown to occur in mouse oocytes and early embryos, and subsequently shown to occur in numerous systems by many researchers, including *in vitro* and *in vivo* systems for a variety of mammals, including human cell lines, and that the Examiner has provided no evidence that the *in vitro* muting shown in rodent cell lines does not correlate to *in vivo* muting in rodents, Applicants respectfully submit that claim 69 is enabled by the examples provided in the specification.

The Examiner also states that "the claims do not specifically recite screening as a means of identifying (see Office Action, p. 5, lines 3-4) and that the application "teaches away from an anti-sense mediated mechanism for muting" (id., p. 6, lines 7-8) and so there is no support for a mechanism of muting that occurs through small dsRNA molecules. First, Applicants point out that the claims now do specifically recited screening as a means of identifying sequences of muting nucleic acid composition. Applicants appreciate the Examiner's helpful guidance in the area of identifying muting nucleic acid compositions, and respectfully submit that the amended claims which now include screening to identify, obviate most of the enablement rejections presented by the Examiner. In the presently amended version of the claims, only those nucleic acid compositions which meet the requirements for muting, whether at the level of post-transcription or transcription, will be identified as muting nucleic acid compositions. Therefore there is no requirement *a priori* for detailed information concerning the exact sequence or nature of the muting sequences -- such sequences will be identified by screening for those which mute the target endogenous gene according to the desired paramaters.

Second, Applicants respectfully point out that the section of the specification which the Examiner cites to support the contention that the muting mechanism does not support the involvement of small dsRNA molecules has been mis-interpreted and mis-cited. What the application actually says in the first two sentences on p. 30 begins on p. 29 with the description of the particular experiment discussed, which specifically looked at muting relative to the initial 585 bp of the gene – i.e. the 5'-end of the gene. The end of p. 29 specifically predicates the conclusion about no antisense involvement discussed

on p. 30 with the caveat that it applies only *in this experiment*, a fact borne out by the title of Example 13 "Pro- α1(I)collagen gene muting is not regulated by differential antisense RNA synthesis *complementary to the initial 585 bp of the gene*" and other statements on p. 15, lines 1-3 which state "Further examples herein indicate that the muting phenomena are not regulated by synthesis of antisense pro-α(I) collagen mRNA synthesis *complementary to the 5' portion of the gene*." Emphasis added. Applicants respectfully submit that there is nothing in the application that precludes *all* antisense RNA arising from other regions of the gene as possibly being involved in the muting mechanism.

The Examiner maintains that because the evidence of record has only provided working examples where DNA sequences were used as muting sequences there is no enablement for other muting nucleic acid compositions, such as RNA or nucleic acid analogs. Applicants again point to MPEP, §2164.08, wherein a Federal Circuit court held that "Not everything necessary to practice the invention need be disclosed. In fact, what is well-known is best omitted." (citing *In re Buchner*, 929 F.2d 660 (Fed. Cir.1991)). Further, the scope of the enablement must bear only "a reasonable correlation" to the scope of the claims (citing *In re Fisher*, 427 F.2d 833 (CCPA 1970)). In the present application, Applicants respectfully submit that the replacement of DNA sequences with DNA analog sequences or sequences of RNA is both well-known and simple to the point of not requiring expertise in that both the RNA and nucleic acid analogs can be readily purchased. Screening to determine whether purchased nucleic acid analogs or purchased RNA (or in vitro transcribed RNA) mutes an endogenous gene is not difficult to perform or imagine performing. There is no need to state that nucleic acid analogs and/or RNA would be purchased (or transcribed) since it is obvious to anyone even remotely

associated with the relevant art that one can purchase nucleic acids and analogs from numerous sources. Further, whereas it is not necessary to provide details for practicing PCR technology when it is listed as a detection or quantitation means because such information is readily available, similarly it is not necessary to provide an express statement of protocol that nucleic acids other than DNA will be purchased or transcribed since companies which sell nucleic acids and analogs, and protocols for transcribing RNA, have been commonplace in the general field of biochemistry since at least the mid-eighties, nearly 30 years!

Applicants respectfully submit that with the inclusion of screening to identify muting nucleic acid compositions, and the general availability of DNA, RNA, and nucleic acid analogs and their ease of manipulation, the present claims are enabled in that respect. Further, as stated above, Applicants appreciate the Examiner's for inclusion of such a step in the claims to help overcome the enablement rejection. Therefore, claims 11, 57, 69 and relevant new claims now include such a step. In light of this amendment, Applicants respectfully submit that it is not necessary to provide "guidance that correlates muting of endogenous genes with nucleic acid molecules other than DNA because screening will identify which "other nucleic acids" mute.

35 U.S.C. § 112, para. 1 – New Matter

As herein amended, Applicants respectfully submit that the new matter rejections of claims 11, 57 and 69 (and relevant dependent claims) should be withdrawn. The claims are amended to require the muting nucleic acid composition to be "double-

stranded" and to require a step for screening to identify a muting nucleic acid composition. Further, claim 69 is amended to encompass only the particular animal cell systems that correlate to the cell systems used in the examples provided.

35 U.S.C. § 1112, para. 2 Rejections

Claims 11, 13-18, 22-24 and 57-69 are rejected for reasons of indefiniteness because of a lack of proper antecedent basis in a) for the element recited in b) in independent claims 11, 57 and 69 (68 being cancelled). Independent claims 11, 57 and 69 are herein amended to correct the antecedent basis issue such that "muting nucleic acid" in b) now reads "muting nucleic acid composition" and has antecedent basis in a) which requires "screening to identify a muting nucleic acid composition." Therefore, Applicants respectfully submit that the claims are no longer indefinite.

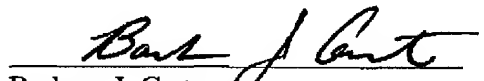
CONCLUSION

For the reasons stated above, it is respectfully submitted that all pending claims are in condition for allowance. Reconsideration of the claims, consideration of the added claims, and a notice of allowance is therefore requested.

It is believed that no extension of time is needed. If any additional fees are required for the timely consideration of this application, however, please charge deposit account number 19-4972. The Examiner is requested to telephone the undersigned if any matters remain outstanding so that they may be resolved expeditiously.

Date: August 6, 2003

Respectfully submitted,



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Clean Version of Pending Claims

11. (currently amended) A method for muting expression of an endogenous gene in a cultured population of animal cells, the method comprising:

(a) screening to identify a muting nucleic acid composition having a sequence that is homologous to a sequence in the endogenous gene, the nucleic acid composition being double stranded, wherein screening to identify comprises the steps of:

(i) designating the entire gene sequence as a potential muting nucleic acid composition;

(ii) identifying a first sequence of muting nucleic acid composition homologous to portions of the endogenous gene that mutes at the level of post-transcription;

(b) delivering the first sequence of muting nucleic acid composition into the population of cells; and

(c) muting expression of the endogenous gene wherein such muting is independent of integration or level of expression of the delivered nucleic acid composition.

14. (currently amended) A method according to claim 11, wherein the first sequence of muting nucleic acid composition is DNA.

57. (currently amended) A method for muting expression of an endogenous gene in a cultured population of animal cells, the method comprising:

(a) screening to identify a muting nucleic acid composition having a sequence that is homologous to a sequence in the endogenous gene, wherein the gene is one of a

collagen, tumor necrosis factor (TNF), *tat*, and an immunoglobulin gene, the nucleic acid composition being double stranded, wherein screening to identify comprises the steps of:

(i) designating the entire gene sequence as a potential muting nucleic acid composition;

(ii) identifying a first sequence of muting nucleic acid composition omologous to portions of the endogenous gene that mutes at the level of post-transcription;

(b) delivering the first sequence of muting nucleic acid composition into the population of cells; and

(c) muting expression of the endogenous gene wherein such muting is independent of integration or level of expression of the delivered nucleic acid composition.

58. (previously added) A method according to claim 57, wherein the endogenous gene is a type I collagen.

59. (previously added) A method according to claim 58, wherein the endogenous gene is pro-α1(I) collagen.

60. (currently amended) A method according to claim 57, wherein the cultured population of animal cells is a population of rodent cells.

69. (currently amended) A method for muting expression of an endogenous gene in a population of rodent cells, the method comprising:

(a) screening to identify a muting nucleic acid composition of DNA having a sequence that is homologous to a sequence in the endogenous gene, wherein the

gene is a collagen gene, the muting nucleic acid composition being double stranded, and wherein screening to identify comprises the steps of:

(i) designating the entire gene sequence as a potential muting nucleic acid composition;

(ii) identifying a first sequence of muting nucleic acid composition homologous to portions of the endogenous gene that mutes at the level of post-transcription;

(b) delivering the first sequence of muting nucleic acid composition into the population of rodent cells; and

(c) muting expression of the endogenous gene wherein such muting is independent of integration or level of expression of the delivered nucleic acid composition.

70. (new) A method for muting according to claim 11 further comprising:

after identifying a first sequence,

(iii) identifying a second sequence of muting nucleic acid composition that mutes at the level of transcription, wherein the first and second muting sequences may be part of a single nucleic acid composition; and

in the delivering, further comprising:

delivering the second sequence of muting nucleic acid composition into the population of cells.

71. (new) A method for muting according to claim 57 further comprising:

after identifying a first sequence,

(iii) identifying a second sequence of muting nucleic acid composition that mutes at the level of transcription, wherein the first and second muting sequences may be part of a single nucleic acid composition; and

in the delivering, further comprising:

delivering the second sequence of muting nucleic acid composition into the population of cells.

72. (new) A method according to claim 71, wherein the endogenous gene is pro-α1(I) collagen and wherein the first or second muting sequence of nucleic acid composition is homologous to an endogenous sequence comprising a portion of the pro-α1(I) collagen gene selected from at least one of the group of: a 5'-untranscribed portion, a transcribed portion, a 3'-untranslated portion, a 3'-untranscribed portion, and a portion that overlaps adjacent ends of at least two portions of the pro-α1(I) collagen gene.

73. (new) A method according to claim 72, wherein the first or second muting sequence of nucleic acid composition comprises a sequence homologous to an endogenous sequence located in a 5'-portion of the pro-α1(I) collagen gene.

74. (new) A method according to claim 72, wherein the first sequence of muting nucleic acid composition comprises a sequence that is homologous to an endogenous sequence located in a 3'-portion of the pro-α1(I) collagen gene including a 3'-untranscribed portion, a 3'-untranslated portion, and a portion that overlaps the 3'-end of a coding portion.

75. (new) A method according to claim 71, wherein delivering the first or second muting sequence of nucleic acid composition is selected from the group of: transforming,

transfecting, electroporating, infecting, or lipofecting as the means for delivering the nucleic acid composition into the cells.

76. (new) A method according to claim 71 wherein the first and/or second sequence of muting nucleic acid composition is DNA.

77. (new) A method for muting according to claim 69 further comprising:

after identifying a first sequence,

(iii) identifying a second sequence of muting nucleic acid composition of DNA that mutes at the level of transcription, wherein the first and second sequence of muting nucleic acid composition may be part of a single DNA composition; and

in the delivering, further comprising:

delivering the second sequence of muting nucleic acid composition into the population of rodent cells.

78. (new) A method according to claim 77, wherein the endogenous gene is a type I collagen.

79. (new) A method according to claim 78, wherein the endogenous gene is pro-α1(I) collagen.

80. (new) A method for muting expression of an endogenous gene in a cultured population of rodent cells, the method comprising:

(a) screening to identify a muting nucleic acid composition having a sequence that is homologous to a sequence in the endogenous gene, wherein the gene is one of a collagen, tumor necrosis factor (TNF), *tat*, and an immunoglobulin gene, the nucleic acid composition being double stranded, wherein screening to identify comprises the steps of:

(i) designating the entire gene sequence as a potential muting nucleic acid composition;

(ii) identifying a first sequence of muting nucleic acid composition homologous to portions of the endogenous gene that mutes at the level of post-transcription;

(b) delivering the first sequence of muting nucleic acid composition into the population of cells; and

(c) muting expression of the endogenous gene wherein such muting is independent of integration or level of expression of the delivered nucleic acid composition.

81. (new) A method for muting according to claim 80 further comprising:
after identifying a first sequence,

(iii) identifying a second sequence of muting nucleic acid composition that mutes at the level of transcription, wherein the first and second muting sequences may be part of a single nucleic acid composition; and
in the delivering, further comprising:

delivering the second sequence of muting nucleic acid composition into the population of cells.

82. (new) A method according to claim 81 wherein the first and second sequence of muting nucleic acid composition is DNA.

83. (new) A method according to claim 81, wherein the endogenous gene is a type I collagen.

84. (new) A method according to claim 83, wherein the endogenous gene is pro-α1(I) collagen.

Staining specificity was controlled by single staining, as well as by using odies in the absence of the primary stain.

n of target cells

cells displaying a membrane-integral version of either wild-type HEL or a mutant exhibiting reduced affinity for HyHEL10 ([R[21], D[101], G[102], N[103]] designated HEL*) were generated by transfecting mouse J558L plasmacytoma cells with constructs analogous to those used[16] for expression of soluble HEL/HEL*, except that 14 Ser/Gly codons, the H2K[b] transmembrane region, and a 23-codon cytoplasmic domain were inserted immediately upstream of the termination codon by polymerase chain reaction. For mHEL–GFP, we included the EGFP coding domain in the Ser/Gly linker. Abundance of surface HEL was monitored by flow cytometry and radiolabelled-antibody binding using HyHEL5 and D1.3 HEL-specific monoclonal antibodies, for which the mutant HELs used in this work show unaltered affinities[10].

Interaction assays

For B-cell/target interaction assays, splenic B cells from 3–83 or MD4 transgenic mice[24,25] carrying (IgM + IgD) BCRs specific for HEL or H2K[k]/H2K[b] were freshly purified on Lympholyte and incubated with a twofold excess of target cells in RPMI, 50 mM HEPES pH7.4, for the appropriate time at 37 °C before being applied to polylysine-coated slides. Cells were fixed in 4% paraformaldehyde/PBS or methanol and permeabilized with PBS/0.1% Triton X-100 before immunofluorescence. We acquired confocal images using a Nikon E800 microscope attached to BioRad Radiance Plus scanning system equipped with 488-nm and 543-nm lasers, as well as differential interference contrast for transmitted light. GFP fluorescence in living cells in real time was visualized using a Radiance 2000 and Nikon E300 inverted microscope. Images were processed using BioRad Lasersharp 1024 or 2000 software to provide single plane images, confocal projections or slicing.

Antigen presentation

Presentation of HEL epitopes to T-cell hybridomas 2G7 (specific for I-E[k][HEL[1–16]]) and 1E5 (specific for I-E[d][HEL[106–116]]) by transfectants of the LK35.2 B-cell hybridoma expressing an HEL-specific IgM BCR was monitored as described[10].

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Appendix A

Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells

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RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene[1–4]. The mediators of sequence-specific messenger RNA degradation are 21- and 22-nucleotide small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from longer dsRNAs[5–9]. Here we show that 21-nucleotide siRNA duplexes specifically suppress expression of endogenous and heterologous genes in different mammalian cell lines, including human embryonic kidney (293) and HeLa cells. Therefore, 21-nucleotide siRNA duplexes provide a new tool for studying gene function in mammalian cells and may eventually be used as gene-specific therapeutics.

Uptake of dsRNA by insect cell lines has previously been shown to 'knock-down' the expression of specific proteins, owing to sequence-specific, dsRNA-mediated mRNA degradation[6,10–12]. However, it has not been possible to detect potent and specific RNA interference in commonly used mammalian cell culture systems, including 293 (human embryonic kidney), NIH/3T3 (mouse fibroblast), BHK-21 (Syrian baby hamster kidney), and CHO-K1 (Chinese hamster ovary) cells, applying dsRNA that varies in size between 38 and 1,662 base pairs (bp)[10,12]. This apparent lack of RNAi in mammalian cell culture was unexpected, because RNAi exists in mouse oocytes and early embryos[13,14], and because RNAi-related, transgene-mediated co-suppression was also observed in cultured Rat-1 fibroblasts[15]. But it is known that dsRNA in the cytoplasm of mammalian cells can trigger profound physiological

reactions that lead to the induction of interferon synthesis[16]. In the interferon response, dsRNA > 30 bp binds and activates the protein kinase PKR[17] and 2′,5′-oligoadenylate synthetase (2′,5′-AS)[18]. Activated PKR stalls translation by phosphorylation of the translation initiation factors eIF2α, and activated 2′,5′-AS causes mRNA degradation by 2′,5′-oligoadenylate-activated ribonuclease L. These responses are intrinsically sequence-nonspecific to the inducing dsRNA.

Base-paired 21- and 22-nucleotide (nt) siRNAs with overhanging 3′ ends mediate efficient sequence-specific mRNA degradation in lysates prepared from *Drosophila* embryos[9]. To test whether siRNAs are also capable of mediating RNAi in cell culture, we synthesized 21-nt siRNA duplexes with symmetric 2-nt 3′ overhangs directed against reporter genes coding for sea pansy (*Renilla reniformis*, RL) and two sequence variants of firefly (*Photinus pyralis*, GL2 and GL3) luciferases (Fig. 1a, b). The siRNA duplexes were co-transfected with the reporter plasmid combinations pGL2/pRL or pGL3/pRL, into *Drosophila* S2 cells or mammalian cells using cationic liposomes. Luciferase activities were determined 20 h after transfection. In *Drosophila* S2 cells (Fig. 2a and b), the specific inhibition of luciferases was complete and similar to results previously obtained for longer dsRNAs[6,10,12,19]. In mammalian cells, where the reporter genes were 50- to 100-fold more strongly expressed, the specific suppression was less complete (Fig. 2c–j). In NIH/3T3, monkey COS-7 and Hela S3 cells (Fig. 2c–h), GL2 expression was reduced 3- to 12-fold, GL3 expression 9- to 25-fold, and RL expression 2- to 3-fold, in response to the cognate siRNAs. For 293 cells, targeting of RL luciferase by RL siRNAs was ineffective, although GL2 and GL3 targets responded specifically (Fig. 2i and j). The lack of reduction of RL expression in 293 cells may be because of its expression, 5- to 20-fold higher than any other mammalian cell line tested and/or to limited accessibility of the target sequence due to RNA secondary structure or associated proteins. Nevertheless, specific targeting of GL2 and GL3 luciferase by the cognate siRNA duplexes indicated that RNAi is also functioning in 293 cells.

The 2-nucleotide 3′ overhang in all siRNA duplexes was composed of (2′-deoxy) thymidine, except for uGL2, which contained

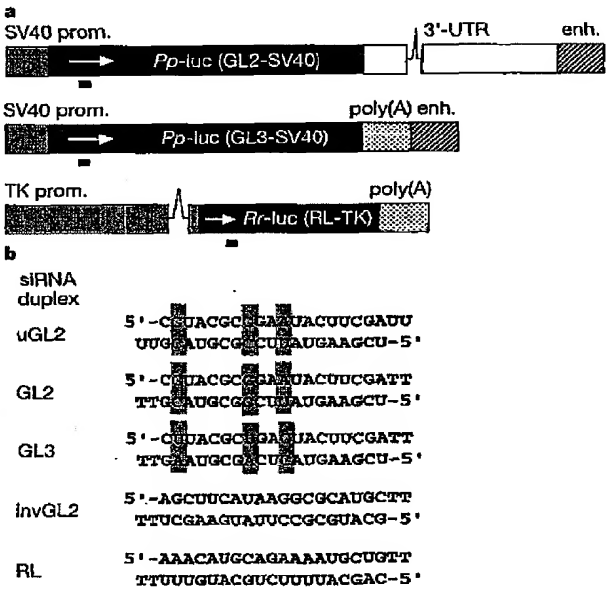

Figure 1 Reporter constructs and siRNA duplexes. **a**, The firefly (*Pp*-luc) and sea pansy (*Rr*-luc) luciferase reporter-gene regions from plasmids pGL2-Control, pGL3-Control, and pRL-TK (Promega) are illustrated; simian virus 40 (SV40) promoter (prom.); SV40 enhancer element (enh.); SV40 late polyadenylation signal (poly(A)); herpes simplex virus (HSV) thymidine kinase promoter, and two introns (lines) are indicated. The sequence of GL3 luciferase is 95% identical to GL2, but RL is completely unrelated to both. Luciferase expression from pGL2 is approximately 10-fold lower than from pGL3 in transfected mammalian cells. The region targeted by the siRNA duplexes is indicated as black bar below the coding region of the luciferase genes. **b**, The sense (top) and antisense (bottom) sequences of the siRNA duplexes targeting GL2, GL3, and RL luciferase are shown. The GL2 and GL3 siRNA duplexes differ by only three single-nucleotide substitutions (boxed in grey). As nonspecific control, a duplex with the inverted GL2 sequence, invGL2, was synthesized. The 2-nucleotide 3′ overhang of 2′-deoxythymidine is indicated as TT; uGL2 is similar to GL2 siRNA but contains ribo-uridine 3′ overhangs.

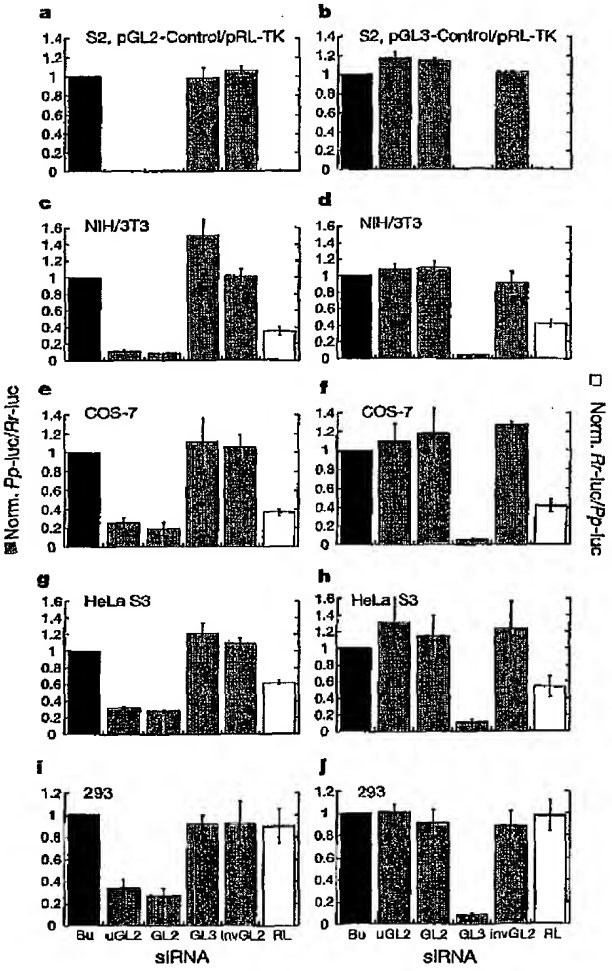

Figure 2 RNA interference by siRNA duplexes. Ratios of target to control luciferase were normalized to a buffer control (Bu, black bars); grey bars indicate ratios of *Photinus pyralis* (*Pp*-luc) GL2 or GL3 luciferase to *Renilla reniformis* (*Rr*-luc) RL luciferase (left axis), white bars indicate RL to GL2 or GL3 ratios (right axis). **a**, **c**, **e**, **g** and **i**, Experiments performed with the combination of pGL2-Control and pRL-TK reporter plasmids; **b**, **d**, **f**, **h** and **j**, experiments performed with the combination of pGL3-Control and pRL-TK reporter plasmids. The cell line used for the interference experiment is indicated at the top of each plot. The ratios of *Pp*-luc/*Rr*-luc for the buffer control (Bu) varied between 0.5 and 10 for pGL2/pRL, and between 0.03 and 1 for pGL3/pRL, respectively, before normalization and between the various cell lines tested. The plotted data were averaged from three independent experiments ± s.d.

uridine residues. The thymidine overhang was chosen because it reduces costs of RNA synthesis and may enhance nuclease resistance of siRNAs in the cell culture medium and within transfected cells. As in the *Drosophila in vitro* system (data not shown), substitution of uridine by thymidine in the 3′ overhang was well tolerated in cultured mammalian cells (Fig. 2a, c, e, g and i), and the sequence of the overhang appears not to contribute to target recognition[9].

In co-transfection experiments, 25 nM siRNA duplexes were used (Figs 2 and 3; concentration is in respect to the final volume of tissue culture medium). Increasing the siRNA concentration to 100 nM did not enhance the specific silencing effects, but started to affect transfection efficiencies, perhaps due to competition for liposome encapsulation between plasmid DNA and siRNA (data not shown). Decreasing the siRNA concentration to 1.5 nM did not reduce the specific silencing effect (data not shown), even though the siRNAs were now only 2- to 20-fold more concentrated than the DNA plasmids; the silencing effect only vanishes completely if the siRNA concentration was dropped below 0.05 nM. This indicates that siRNAs are extraordinarily powerful reagents for mediating gene silencing, and that siRNAs are effective at concentrations that are several orders of magnitude below the concentrations applied in conventional antisense or ribozyme gene-targeting experiments[20].

To monitor the effect of longer dsRNAs on mammalian cells, 50- and 500-bp dsRNAs that are cognate to the reporter genes were prepared. As a control for nonspecific inhibition, dsRNAs from humanized GFP (hG)[21] was used. In these experiments, the reporter plasmids were co-transfected with either 0.21 μg siRNA duplexes or 0.21 μg longer dsRNAs. The siRNA duplexes only reduced the expression of their cognate reporter gene, while the longer dsRNAs strongly and nonspecifically reduced reporter-gene expression. The effects are illustrated for HeLa S3 cells as a representative example (Fig. 3a and b). The absolute luciferase activities were decreased nonspecifically 10- to 20-fold by 50-bp dsRNA, and 20- to 200-fold by 500-bp dsRNA co-transfection, respectively. Similar nonspecific effects were observed for COS-7 and NIH/3T3 cells. For 293 cells, a 10- to 20-fold nonspecific reduction was observed only for 500-bp dsRNAs. Nonspecific reduction in reporter-gene expression by dsRNA > 30 bp was expected as part of the interferon response[16]. Interestingly, superimposed on the nonspecific interferon response, we detect additional sequence-specific, dsRNA-mediated silencing. The sequence-specific silencing effect of long dsRNAs, however, became apparent only when the relative reporter-gene activities were normalized to the hG dsRNA controls (Fig. 3c). Sequence-specific silencing by 50- or 500-bp dsRNAs reduced the targeted reporter-gene expression by an additional 2- to 5-fold. Similar effects were also detected in the other three mammalian cell lines tested (data not shown). Specific silencing effects with dsRNAs (356–1,662 bp) were previously reported in CHO-K1 cells, but the amounts of dsRNA required to detect a 2- to 4-fold specific reduction were about 20-fold higher than in our experiments[12]. Also, CHO-K1 cells appear to be deficient in the interferon response. In another report, 293, NIH/3T3 and BHK-21 cells were tested for RNAi using luciferase/β-galactosidase (lacZ) reporter combinations and 829-bp specific lacZ or 717-bp nonspecific green fluorescent protein (GFP) dsRNA[10]. The lack of detected RNAi in this case may be due to the less sensitive luciferase/lacZ reporter assay and the length differences of target and control dsRNA. Taken together, our results indicate that RNAi is active in mammalian cells, but that the silencing effect is difficult to detect if the interferon system is activated by dsRNA > 30 bp.

To test for silencing of endogenous genes, we chose four genes coding for cytoskeletal proteins: lamin A/C, lamin B1, nuclear mitotic apparatus protein (NuMA) and vimentin[27]. The selection was based on the availability of antibodies needed to quantitate the silencing effect. Silencing was monitored 40 to 45 h after transfection to allow for turnover of the protein of the targeted genes. As shown in Fig. 4, the expression of lamin A/C was specifically reduced by the cognate siRNA duplex (Fig. 4a), but not when nonspecific siRNA directed against firefly luciferase (Fig. 4b) or buffer (Fig. 4c) was used. The expression of a non-targeted gene, NuMA, was unaffected in all treated cells (Fig. 4d–f), demonstrating the integrity of the targeted cells. The reduction in lamin A/C proteins was more than 90% complete as quantified by western blotting (Fig. 4j, k). We note that lamin A/C 'knock-out' mice are

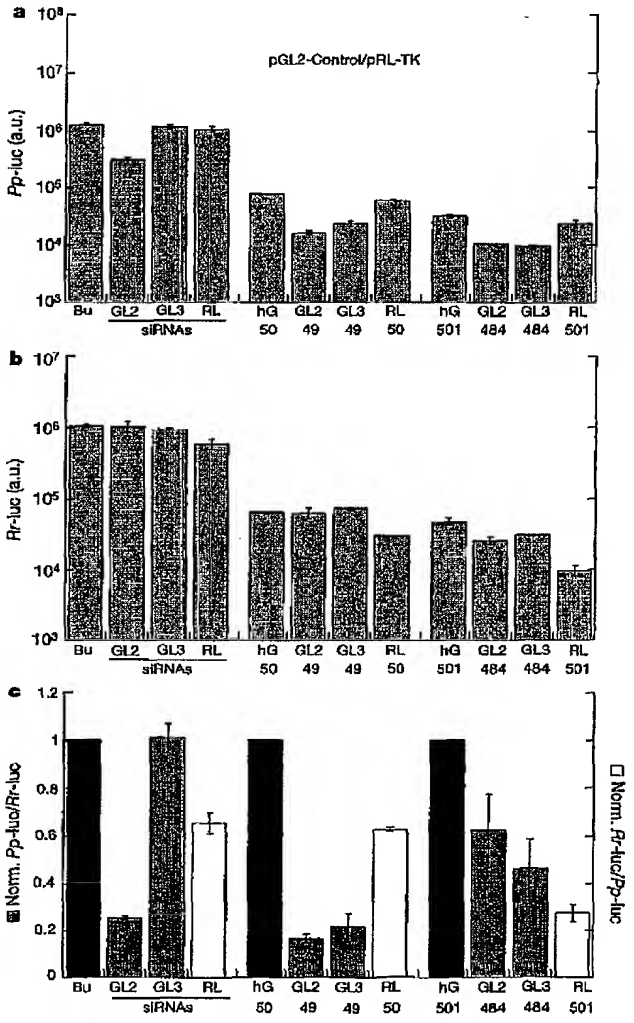

Figure 3 Effects of 21-nucleotide siRNAs, 50-bp, and 500-bp dsRNAs on luciferase expression in HeLa cells. The exact length of the long dsRNAs in base pairs is indicated below the bars. Experiments were performed with pGL2-Control and pRL-TK reporter plasmids. The data were averaged from two independent experiments ± s.d. **a**, Absolute *Pp*-luc expression, plotted in arbitrary luminescence units (a.u.). **b**, *Rr*-luc expression, plotted in arbitrary luminescence units. **c**, Ratios of normalized target to control luciferase. The ratios of luciferase activity for siRNA duplexes were normalized to a buffer control (Bu, black bars); the luminescence ratios for 50- or 500-bp dsRNAs were normalized to the respective ratios observed for 50- and 500-bp dsRNAs from humanized GFP (hG, black bars). We note that the overall differences in sequence between the 49- and 484-bp GL2 and GL3 dsRNAs are not sufficient to confer specificity for targeting GL2 and GL3 targets (43-nucleotide uninterrupted identity in 49-bp segment, 239-nucleotide longest uninterrupted identity in 484-bp segment)[30].

viable for a few weeks after birth[23] and that the lamin A/C knockdown in cultured cells was not expected to cause cell death. Lamin A and C are produced by alternative splicing in the 3′ region and are present in equal amounts in the lamina of mammalian cells (Fig. 4j, k). Transfection of siRNA duplexes targeting lamin B1 and NuMA reduced the expression of these proteins to low levels (data not shown), but we were not able to observe a reduction in vimentin expression. This could be due to the high abundance of vimentin in the cells (several per cent of total cell mass) or because the siRNA sequence chosen was not optimal for targeting of vimentin.

The mechanism of the 21-nucleotide siRNA-mediated interference process in mammalian cells remains to be uncovered, and silencing might occur post-transcriptionally and/or transcriptionally. In *Drosophila* lysate, siRNA duplexes mediate post-transcriptional gene silencing by reconstitution of siRNA-protein complexes (siRNPs), which guide mRNA recognition and targeted cleavage[6,7,9]. In plants, dsRNA-mediated post-transcriptional silencing has also been linked to DNA methylation, which may also be directed by 21-nucleotide siRNAs[24]. Methylation of promoter regions can lead to transcriptional silencing[25], but methylation in coding sequences does not[26]. DNA methylation and transcriptional silencing in mammals are well documented processes[27], yet their mechanisms have not been linked to that of post-transcriptional silencing. Methylation in mammals is predominantly directed towards CpG dinucleotide sequences. There is no CpG sequence in the RL or lamin A/C siRNA, although both siRNAs mediate specific silencing in mammalian cell culture, so it is unlikely that DNA methylation is essential for the silencing process.

Thus we have shown, for the first time, siRNA-mediated gene silencing in mammalian cells. The use of exogenous 21-nucleotide siRNAs holds great promise for analysis of gene function in human cell culture and the development of gene-specific therapeutics. It will also be of interest in understanding the potential role of endogenous siRNAs in the regulation of mammalian gene function. □

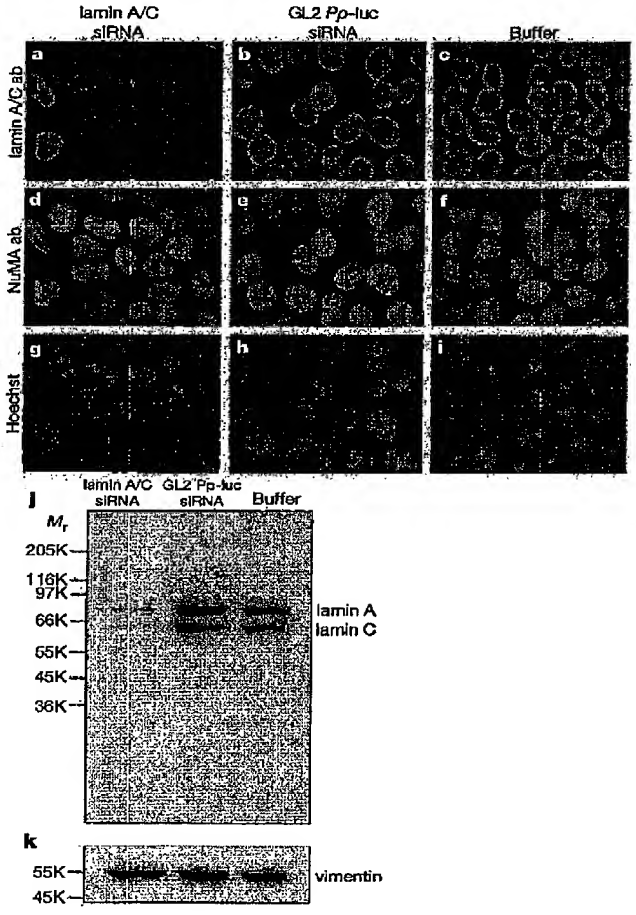

Figure 4 Silencing of nuclear envelope proteins lamin A/C in HeLa cells. Triple fluorescence staining of cells transfected with lamin A/C siRNA duplex (**a**, **d**, **g**), with GL2 luciferase siRNA duplex (nonspecific siRNA control) (**b**, **e**, **h**), and with buffer only (**c**, **f**, **i**). **a**–**c**, Staining with lamin A/C specific antibody; **d**–**f**, staining with NuMA-specific antibody; **g**–**i**, Hoechst staining of nuclear chromatin. Bright fluorescent nuclei in **a** represent untransfected cells. **j**, **k**, Western blots of transfected cells using lamin A/C- (**j**) or vimentin-specific (**k**) antibodies. The Western blot was stripped and re-probed with vimentin antibody to check for equal loading of total protein.

Methods

RNA preparation

21-nucleotide RNAs were chemically synthesized using Expedite RNA phosphoramidites and thymidine phosphoramidite (Proligo, Germany). Synthetic oligonucleotides were deprotected and gel-purified[9]. The accession numbers given below are from GenBank. The siRNA sequences targeting GL2 (Acc. No. X65324) and GL3 luciferase (Acc. No. U47296) corresponded to the coding regions 153–173 relative to the first nucleotide of the start codon; siRNAs targeting RL (Acc. No. AF025846) corresponded to region 119–139 after the start codon. The siRNA sequence targeting lamin A/C (Acc. No. X03444) was from position 608–630 relative to the start codon; lamin B1 (Acc. No. NM_005573) siRNA was from position 672–694; NuMA (Acc. No. Z11583) siRNA from position 3,988–4,010, and vimentin (Acc. No. NM_003380) from position 346–368 relative to the start codon. Longer RNAs were transcribed with T7 RNA polymerase from polymerase chain reaction (PCR) products, followed by gel purification. The 49- and 484-bp GL2 or GL3 dsRNAs corresponded to positions 113–161 and 113–596, respectively, relative to the start of translation; the 50- and 501-bp RL dsRNAs corresponded to position 118–167 and 118–618, respectively. PCR templates for dsRNA synthesis targeting humanized GFP (hG) were amplified from pAD3 (ref. 21), whereby 50- and 501-bp hG dsRNA corresponded to positions 121–170 and 121–621, respectively, to the start codon.

For annealing of siRNAs, 20 μM single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90 °C followed by 1 h at 37 °C. The 37 °C incubation step was extended overnight for the 50- and 500-bp dsRNAs, and these annealing reactions were performed at 8.4 μM and 0.84 μM strand concentrations, respectively.

Cell culture

S2 cells were propagated in Schneider's *Drosophila* medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 25 °C. 293, NIH/3T3, HeLa S3, HeLa SS6, COS-7 cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Cells were regularly passaged to maintain exponential growth. Twenty-four h before transfection at 50–80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics ($1–3 \times 10^5$ cells ml^{-1}) and transferred to 24-well plates (500 μl per well). S2 cells were not trypsinized before splitting. Co-transfection of reporter plasmids and siRNAs was carried out with Lipofectamine 2000 (Life Technologies) as described by the manufacturer for adherent cell lines. Per well, 1.0 μg pGL2-Control (Promega) or pGL3-Control (Promega), 0.1 μg pRL-TK (Promega), and 0.21 μg siRNA duplex or dsRNA, formulated into liposomes, were applied; the final volume was 600 μl per well. Cells were incubated 20 h after transfection and appeared healthy thereafter. Luciferase expression was subsequently monitored with the Dual luciferase assay (Promega). Transfection efficiencies were determined by fluorescence microscopy for mammalian cells lines after co-transfection of 1.1 μg hGFP-encoding pAD3 (ref. 21) and 0.21 μg inverted GL2 siRNA, and were 70–90%. Reporter plasmids were amplified in XL-1 Blue (Stratagene) and purified using the Qiagen EndoFree Maxi Plasmid Kit.

Transfection of siRNAs for targeting endogenous genes was carried out using Oligofectamine (Life Technologies) and 0.84 μg siRNA duplex per well, but it was recently found that as little as 0.01 μg siRNAs per well are sufficient to mediate silencing. HeLa SS6 cells were transfected one to three times in approximately 15 h intervals and were assayed 40 to 45 h after the first transfection. It appears, however, that a single transfection is as efficient as multiple transfections. Transfection efficiencies as determined by immunofluorescence of targeted cells were in the range of 90%. Specific silencing of targeted genes was confirmed by at least three independent experiments.

Western blotting and immunofluorescence microscopy

Monoclonal 636 lamin A/C specific antibody[28] was used as undiluted hybridoma supernatant for immunofluorescence and 1/100 dilution for western blotting. Affinity-purified polyclonal NuMA protein 705 antibody[29] was used at a concentration of 10 μg ml^{-1} for

immunofluorescence. Monoclonal V9 vimentin-specific antibody was used at 1/2,000 dilution. For western blotting, transfected cells grown in 24-well plates were trypsinized and harvested in SDS sample buffer. Equal amounts of total protein were separated on 12.5% polyacrylamide gels and transferred to nitrocellulose. Standard immunostaining was carried out using ECL enhanced chemiluminescence technique (Amersham Pharmacia).

For immunofluorescence, transfected cells grown on glass coverslips in 24-well plates were fixed in methanol for 6 min at −10 °C. Target gene specific and control primary antibody were added and incubated for 80 min at 37 °C. After washing in phosphate buffered saline (PBS), Alexa 488-conjugated anti-rabbit (Molecular Probes) and Cy3-conjugated anti-mouse (Dianova) antibodies were added and incubated for 60 min at 37 °C. Finally, cells were stained for 4 min at room temperature with Hoechst 33342 (1 μM in PBS) and embedded in Mowiol 488 (Hoechst). Pictures were taken using a Zeiss Axiophot camera with a Fluar 40/1.30 oil objective and MetaMorph Imaging Software (Universal Imaging Corporation) with equal exposure times for the specific antibodies.

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Ribosomal peptidyl transferase can withstand mutations at the putative catalytic nucleotide

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Peptide bond formation is the principal reaction of protein synthesis. It takes place in the peptidyl transferase centre of the large (50S) ribosomal subunit. In the course of the reaction, the polypeptide is transferred from peptidyl transfer RNA to the α-amino group of amino acyl-tRNA. The crystallographic structure of the 50S subunit showed no proteins within 18 Å from the active site, revealing peptidyl transferase as an RNA enzyme[1]. Reported unique structural and biochemical features of the universally conserved adenine residue A2451 in 23S ribosomal RNA (*Escherichia coli* numbering) led to the proposal of a mechanism of rRNA catalysis that implicates this nucleotide as the principal catalytic residue[2,3]. *In vitro* genetics allowed us to test the importance of A2451 for the overall rate of peptide bond formation. Here we report that large ribosomal subunits with mutated A2451 showed significant peptidyl transferase activity in several independent assays. Mutations at another nucleotide, G2447, which is essential to render catalytic properties to A2451 (refs 2, 3), also did not dramatically change the transpeptidation activity. As alterations of the putative catalytic residues do not severely affect the rate of peptidyl transfer the ribosome apparently promotes transpeptidation not through chemical catalysis, but by properly positioning the substrates of protein synthesis.

The proposed role of A2451 in the peptidyl transfer reaction is

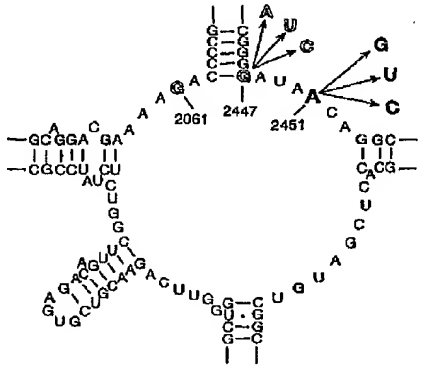

Figure 1 The secondary structure of the central loop of domain V of *T. aquaticus* 23S rRNA. Position A2451 (*E. coli* 23S rRNA numeration), the principal catalytic nucleotide in the proposed general acid–base catalytic mechanism of peptide bond formation[2,3], is shown in bold. Its tertiary interaction partners, guanine residues 2061 and 2447, suggested to be essential for rendering catalytic properties to A2451, are outlined. Arrows indicate the mutations engineered in 23S rRNA.

Appendix B

ed that these find a corresponding target mRNA that they can immediately hop onto (*26*).

The polymerase required for this amplification is probably different in different tissues. In the germline of *C. elegans*, the *ego-1* gene has been implicated in RNAi; it has sequence homology to a factor previously isolated from the tomato as an RdRP (*29*). In somatic *C. elegans* cells, another RdRP homolog has been implicated: *rrf-1*. Mutation of the *rrf-1* gene results in loss of RNAi and in significant decrease of siRNAs. Inactivation of another RdRP homolog has the opposite effect, of enhancing RNAi [*rrf-3* (*11*)]. The *rrf-3* gene product may be less active and may compete with RRF-1 in the relevant complex. In *Dictyostelium*, three RdRP homologs have been described. Loss of one of them, *rrpA*, resulted in loss of RNAi and of detectable siRNAs (*13*).

The *Arabidopsis thaliana* RdRP homolog SDE1/SGS2 is also required for transitive RNAi (*12*). A significant difference between transitive RNAi in *C. elegans* and plants (*Nicotiana bethamiana* and *Arabidopsis*) is that, in plants, the transitive effect can occur in the 3′ as well as the 5′ direction, and as a consequence, secondary siRNAs are found both 5′ and 3′ of the targeted region. In plants, siRNAs may direct an RdRP to an mRNA, triggering unprimed RdRP activity of the complete RNA molecule. Alternatively, the initial reaction may show polarity, but frequent template jumps may occur.

The combination of siRNA stabilization and transitive RNAi results in a "chain reaction," in which multiple cycles of replication can occur, followed by Dicing, new priming, and a new round of amplification (Fig. 1).

Conclusion

We are beginning to dissect an ancient mechanism that protects the most sensitive part of a species: its genetic code. Like the vertebrate immune system, the machinery recognizes molecular parasites, raises an initial response, and stabilizes and amplifies this response. Given the conservation of parts of the RNAi-silencing machinery [see reviews (*30*, *31*)], this genome defense mechanism should be widespread, although details may differ. It is thus also possible that RNAi silencing refers to a family of mechanisms that are quite different in context and detail. This will almost certainly be the case for more specific aspects of the biology: for example systemic RNAi in *C. elegans* (*21*), spreading of silencing in plants (*22*), and suppression of silencing induced by several plant viruses (*32*).

Just as knowledge of immunology has laid the foundation for (experimental) immune therapy, a thorough understanding of the genome's immune system has great potential for applications in directed gene silencing, in experimental biology, and possibly also in disease therapy.

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VIEWPOINT

Ancient Pathways Programmed by Small RNAs

Phillip D. Zamore

Double-stranded RNA can now be used in a wide variety of eukaryotes to suppress the expression of virtually any gene, allowing the rapid analysis of that gene's function, a technique known as RNA interference. But how cells use the information in double-stranded RNA to suppress gene expression and why they contain the machinery to do so remain the subjects of intense scrutiny. Current evidence suggests that RNA interference and other "RNA silencing" phenomena reflect an elaborate cellular apparatus that eliminates abundant but defective messenger RNAs and defends against molecular parasites such as transposons and viruses.

Virtually any gene can now be disrupted in cultured human cells, flies, worms, and a growing list of other organisms in just a week or two (*1*, *2*) using new tools based on the cellular phenomenon of "RNA silencing" (Fig. 1). These new tools likely will soon be extended to whole mammals (*3*–*5*) and may one day form the basis of a new class of drugs to treat human disease. Knowing only the DNA sequence of a gene, molecular biologists can design potent, sequence-specific inhibitors—a form of double-stranded RNA—that block expression of just that gene. Using such inhibitors, we can now ask for each of the tens of thousands of human messenger RNAs (mRNAs) the central question of genetics: what does this gene do?

White Flowers and Silenced Worms

New tools for evaluating gene function (Fig. 1) sprang from the discovery that disparate and bizarre examples of RNA silencing are all man-

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ifestations of the same underlying cellular process. Examples of RNA silencing accumulated throughout the 1990s in fungi, worms, flies, and mice. Common to all of these was that the introduction into cells of nucleic acid bearing sequence from a cellular gene decreased the steady-state level of the corresponding cellular mRNA. RNA silencing was first observed in plants. For example, researchers trying to generate more vividly purple petunias created transgenic plants that harbored an extra copy of the enzyme responsible for purple pigment. Yet the resulting plants often produced white flowers. The production of both the transgenic and the petunia's own purple-making genes was switched off or "cosuppressed" (*6–8*). The transgenic copies of the gene, intended to produce more gene product than is made in nontransgenic plants, surprisingly made less. This phenomenon, posttranscriptional gene silencing (PTGS), is now used to make genetically modified plants that lack specific endogenous gene products. In parallel, researchers working with the nematode *Caenorhabditis elegans* tried to use antisense technology to block the function of a gene (*9*). They injected antisense RNA for the gene into the worm, with the intent that the antisense RNA would pair with the gene's mRNA transcript and block its translation into protein. The experiment was a qualified success. The protein's concentration was indeed reduced, but injecting sense RNA—that is, the mRNA itself—likewise blocked protein production. How sense RNA could block gene expression was not understood. Fire, Mello, and co-workers brought understanding to these observations when they showed that both the antisense and the sense RNA preparations contained contaminating double-stranded RNA and that, in fact, this double-stranded RNA was the real trigger of gene silencing (*10*). This phenomenon, in which experimentally introduced double-stranded RNA leads to loss of the expression of the corresponding cellular gene, is called RNA interference or "RNAi" (*11*).

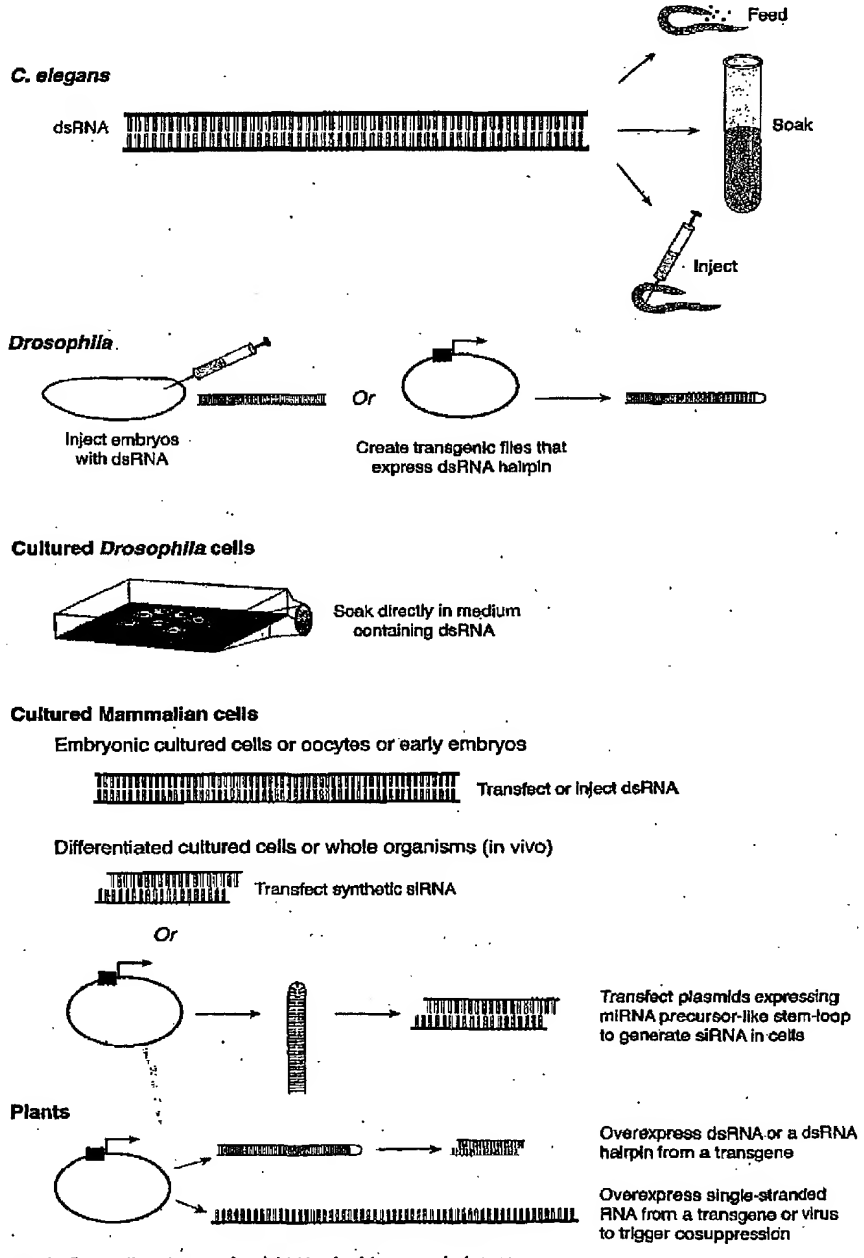

Fig. 1. Gene silencing tools. dsRNA, double-stranded RNA.

We now understand that both PTGS and RNAi are manifestations of a broader group of posttranscriptional RNA silencing phenomena common to virtually all eukaryotes, except perhaps the baker's yeast, *Saccharomyces cerevisiae* (*12*). In all these RNA silencing phenomena, transcription of the silenced gene is unperturbed, yet the mRNA transcript for the gene fails to accumulate to its normal cytoplasmic concentration. That is, the gene is copied into mRNA in the nucleus, but the mRNA is destroyed—probably in the cytoplasm—as quickly as it is made. RNA silencing pathways in protozoa, plants, fungi, and animals require a set of related proteins, suggesting that the common aspects of the pathways are quite ancient (*13–24*). Archea and prokaryotes lack these proteins, so RNA silencing is probably a eukaryotic innovation.

Understanding Started Small

Our understanding of the mechanism of RNA silencing was shaped in large part by the discovery that silenced plants always contain small RNAs, about 25 nucleotides (nt) long, derived from the sequence of the silenced gene. Such small RNAs are never found in plants that do not display silencing (*25*). The small RNAs include both sense and antisense fragments of the silenced gene's sequence. Similar small RNAs are found as part of a ribonuclease complex in extracts of insect cells pretreated with double-stranded RNA and in *Drosophila* embryo lysates that reproduce most, if not all, of the RNAi pathway in a test tube (*26, 27*). We now know that these "small interfering RNAs" or "siRNAs" are double-stranded and that they are chopped from longer double-stranded RNA by an ATP-dependent ribonuclease called "Dicer" (*21, 28*). In flies and humans, siRNAs are

21- to 23-nt-long double-stranded RNAs bearing two-nucleotide, 3′ overhanging ends (*2*, *29*). The peculiar structure of siRNAs reflects the enzymatic mechanism by which Dicer, a member of the RNase III family of double-stranded RNA-specific endonucleases, cleaves double-stranded RNA (*30*). Synthetic siRNAs with the structure of Dicer products are now routinely used to trigger silencing in cultured human cells, providing an alternative to the time-consuming process of making somatic cell knockouts (*1*, *2*). Data from in vitro studies in *Drosophila* suggest that the siRNAs produced by Dicer are then transferred to a second enzyme complex, the RNA-induced silencing complex (RISC), which contains an endoribonuclease that is distinct from Dicer (*20*, *26*, *28*). The endoribonuclease uses the sequence encoded by the antisense siRNA strand to find and destroy mRNAs of complementary sequence. The siRNA thus acts as a guide, restricting the ribonuclease to cleave only RNAs complementary to one of the two siRNA strands. How the RNA-degrading capacity of the ribonuclease is constrained by the siRNA guide is not yet understood, but current evidence suggests that it cuts the mRNA partner of an siRNA-mRNA duplex across from the center of the siRNA (*29*, *31*). It remains to be determined whether the RISC is a bona fide enzyme in which a single siRNA molecule directs multiple rounds of mRNA cleavage.

So has the once bizarre collection of disparate RNA silencing phenomena been stripped of its mystery? In a way, yes. RNA silencing in plants (PTGS or cosuppression), fungi ("quelling"), and animals (RNAi) now share a common intellectual framework, united by common genes (discovered, for the most part, by classical genetics) and organized around a stepwise pathway that continues to emerge from biochemical studies of RNAi in *Drosophila* and human cells. But the mystery has also intensified, as the molecular dissection of RNA silencing phenomena reveals differences in mechanism between organisms and between different "triggers" of silencing, of which double-stranded RNA is but one. The mystery has also intensified as we identify—but do not yet understand—the connections between RNA silencing and the normal functions of the eukaryotic cell, especially between RNA silencing and animal development [reviewed in (*32*, *33*)].

One Mechanism or Variations on a Theme?

Experiments in *C. elegans* suggest that RNAi requires a target RNA copying step, without which siRNAs fail to reach sufficient concentration to accomplish target mRNA cleavage (*34*). These studies, as well as similar studies in plants and fungi (*13*, *18*, *35*), demonstrate a clear genetic role for a family of RNA-dependent RNA polymerases (RdRPs) in the mechanism of RNA silencing. Furthermore, the *Arabidopsis* RdRP, *SDE-1/SGS-2*, is required for PTGS but is dispensable for the silencing of viruses that encode their own RdRP proteins. Copying of the target mRNA into double-stranded RNA could also explain why stable accumulation of siRNA duplexes in *Dictyostelium discoideum* requires the presence of both the target mRNA and a gene encoding a putative RdRP (*23*). A high concentration of siRNA may be achieved in vivo by copying the target RNA into new double stranded RNA, which is then diced into a new crop of siRNAs. In this view, exogenous double-stranded RNA does not produce enough siRNA-programmed RISC complexes to accomplish silencing. Instead, the exogenous double strand is proposed to be diced into "primary" siRNAs that function as primers for new double-stranded RNA synthesis. Such synthesis is likely catalyzed by the RdRP using the target mRNA as a transcription template. However, a direct role for primers in the function of the RdRP has not been demonstrated in silencing in any organism, and other mechanisms of RdRP action are possible. The double-stranded RNA synthesized by the RdRP would then be cleaved by Dicer to generate a new crop of "secondary" siRNAs, amplifying the silencing signal and leading to enough RISC complex to establish silencing. Remarkably, secondary—that is, RdRP-dependent—siRNA production in *C. elegans* [but not in *Neurospora* (*36*), *Dictyostelium* (*23*), or *Arabidopsis* (*25*)] is asymmetric, where only the target-complementary siRNA strand can be detected (*37*). At present, we cannot answer the question, why are secondary siRNAs required to eliminate target mRNA in worms, but primary siRNAs appear to suffice in flies and humans?

No member of this nearly ubiquitous family of RdRPs has been detected by BLAST searching the nearly complete genome sequences of *Drosophila melanogaster* or humans. Furthermore, a variety of experiments argue against a role for an RdRP in the RNAi pathway in *Drosophila* (*20*, *27*, *28*, *38*, *39*). In humans, the most compelling evidence against the involvement of an RdRP is the recent finding that siRNAs that cannot act as primers for an RdRP because they contain blocked 3′ termini nonetheless trigger efficient RNAi in vivo (*40*). Why might an RNA-copying enzyme be essential for RNAi in some organisms (*C. elegans*, *Arabidopsis*, *Neurospora*, *Dictyostelium*) but not in others (*Drosophila*, humans)? To begin to answer this question requires an understanding of how cells sense the various RNA-silencing triggers, rather than how they dispatch mRNA targets.

A Diversity of Silencing Triggers

There is strong evidence that RNA silencing phenomena share a common biochemical machinery, but that this machinery likely lies downstream of a more diverse array of sensors that detect different silencing "triggers" (*41*). Double-stranded RNA is but one of several RNA molecules that induce silencing. For example, the white-flowered petunias that overexpressed the purple pigment–making gene did so in response to the introduction of a transgene designed to produce a high level of single-stranded, sense RNA. So why did the flowers silence the transgenic and the endogenous genes? The standard explanation is that the transgene made "aberrant" RNA. It is tempting to view this aberrant RNA as simply unanticipated double-stranded RNA that triggers silencing by the standard mechanism proposed for RNAi (Fig. 2A). But other evidence suggests that aberrant RNA may be single-stranded and that it is converted into double-stranded RNA by cellular enzymes designed to detect its aberrancy. What makes single-stranded RNA aberrant is an unresolved question in our understanding of RNA silencing. Premature termination of transcription, inappropriate pre-mRNA splicing, failure to associate with the appropriate hnRNP proteins, lack of a poly(A)$^+$ tail, or failure to be translated may all make an mRNA aberrant. Common to all of these may be increased access of the RNA to an RdRP that could convert aberrant single-stranded RNA into double-stranded RNA, which could then enter the RNAi pathway through its conversion by Dicer into siRNAs. Thus, the chief candidate for an aberrant RNA sensor is the RdRP. Perhaps *C. elegans* and other organisms that require an RdRP for silencing sense double-stranded RNA by a mechanism that cannot directly load siRNAs into the RISC complex (Fig. 2B). In these organisms, silencing is probably not triggered by creating siRNAs from the exogenous double-stranded RNA, but rather by using the primary siRNAs to activate the pathway that normally senses aberrant RNA, the cosuppression pathway. One testable prediction of this model is that all genes required for cosuppression in worms will be required for RNAi, but not vice versa (*42*, *43*). A second prediction is that cosuppression in flies and, perhaps, mammals will not use the same RdRP-based mechanism thought to operate in worms or plants.

Why should RdRPs be required for RNA silencing in some organisms, such as *C. elegans*, *Arabidopsis*, and *Neurospora*, but not *Drosophila* and human cells? An obvious answer is that an RNA polymerase of similar biochemical activity but different sequence fulfills this function in flies and mammals. A noncanonical RdRP has been proposed to play a role in RNAi in *Drosophila* (*44*), but biochemical evidence does not support an obligatory role for such an enzyme in flies (*28*, *38*) or in human cells (*40*). siRNA-mediated RNAi in human cells is transitory, with cells recovering from a single treatment with siRNAs in 4 to 6 days (*40*, *45*), suggesting that the original siRNAs are not amplified or copied. siRNAs may simply be less stable in some organisms than others. In those organisms in which

siRNAs are acutely unstable, an RdRP might be essential to amplify the original silencing signal, generating secondary siRNAs. It is important to note that this amplification may not involve the primed synthesis of new RNA. Rather, the initial double-stranded RNA may yield a small number of siRNA-programmed RISC endonuclease complexes that cleave the target mRNA. The resulting mRNA fragments, in particular the relatively stable, capped 5′ fragment, might constitute aberrant mRNA, which would be copied into double-stranded RNA by an RdRP in an unprimed reaction (Fig. 2B). A high concentration of such aberrant RNA may be required to activate the RdRP, with the normal products of mRNA turnover at too low a concentration to provoke RdRP-mediated copying. As concentration-dependent sensors of aberrant RNA, RdRP enzymes should be mediocre polymerases, with relatively low affinity for RNA templates and modest processivity, allowing them to ignore healthy, cellular mRNAs. Consistent with this view, Han and Grierson recently showed that, in tomatoes, siRNAs were preferentially produced from the 3′ end of a transgene that triggered silencing but not from the endogenous target RNA that is silenced (*46*). This suggests that the RdRP initiates primer-independent copying at the 3′ end of an abundant but aberrant transcript from the transgene but does not copy the nonaberrant, and presumably less abundant, endogenous mRNA. The double-stranded RNA resulting from RdRP copying of an aberrant transcript would then be converted by Dicer into siRNAs, which, as part of a RISC complex, could destroy additional aberrant RNA from the transgene, as well as transcripts from an endogenous gene of corresponding sequence, leading to the silencing of both transgene and endogenous gene. This explains the observed decline in siRNA levels that accompanies the establishment of silencing in tomatoes (*46*). Furthermore, both a 5′ fragment lacking a poly(A)$^+$ tail and a 3′ polyadenylated fragment of the endogenous, silenced mRNA were detected, additional evidence that the RISC-based pathway of siRNA-directed endonucleolytic cleavage operates in plants, too (*46*). Short antisense RNA fragments may also be silencing triggers, eliciting silencing by recruiting an RdRP to convert an mRNA into double-stranded RNA. Plasterk and co-workers have shown that in *C. elegans* exogenous single-stranded RNA oligomers of as long as 40 nucleotides can trigger silencing (*37*). Remarkably, the genetic requirements for this type of silencing resemble those of cosuppression, not RNAi. An alternative view, of course, is that the pathway worked out in *Drosophila* does not exist in all organisms. It is sobering to recall that RISC activity has not yet been demonstrated in *C. elegans* or *Neurospora*. Nevertheless, recent evidence suggests that siRNAs direct endonucleolytic cleavage of the target RNA in human cells, indicating the presence of a RISC in mammals (*40*).

In plants and in animals, RNAi-like mechanisms defend against viral infection (*47*–*49*). Thus, viral infection is another distinct trigger of silencing. For some RNA viruses, double-stranded intermediates in the viral life cycle may provoke RNAi, but for others, such as DNA viruses, the molecular species that induce viral silencing are yet unidentified. Transposons and repetitive DNA sequences are also kept in check in eukaryotic cells by RNAi-like mechanisms. In *C. elegans*, silencing of such parasitic DNA requires downstream components of the RNAi pathway but does not use the same upstream sensors. For example, the *mut-7* gene is required for RNAi, and worms defective in *mut-7* show increased transposition (*14*, *50*). MUT-7, a putative 3′-to-5′ exonuclease, may actually function far downstream in the pathway, degrading the initial endonucleolytic fragments produced by the RISC. *mut-7* function may be especially important when RISC-mediated cleavage leaves abundant, translatable mRNA fragments. In contrast, no increase in transposition occurs in *rde-1* mutants, which are nonetheless completely refractory to RNAi elicited by exogenous double-stranded RNA (*14*). If double-stranded RNA produced from transposons triggers their silencing, why do

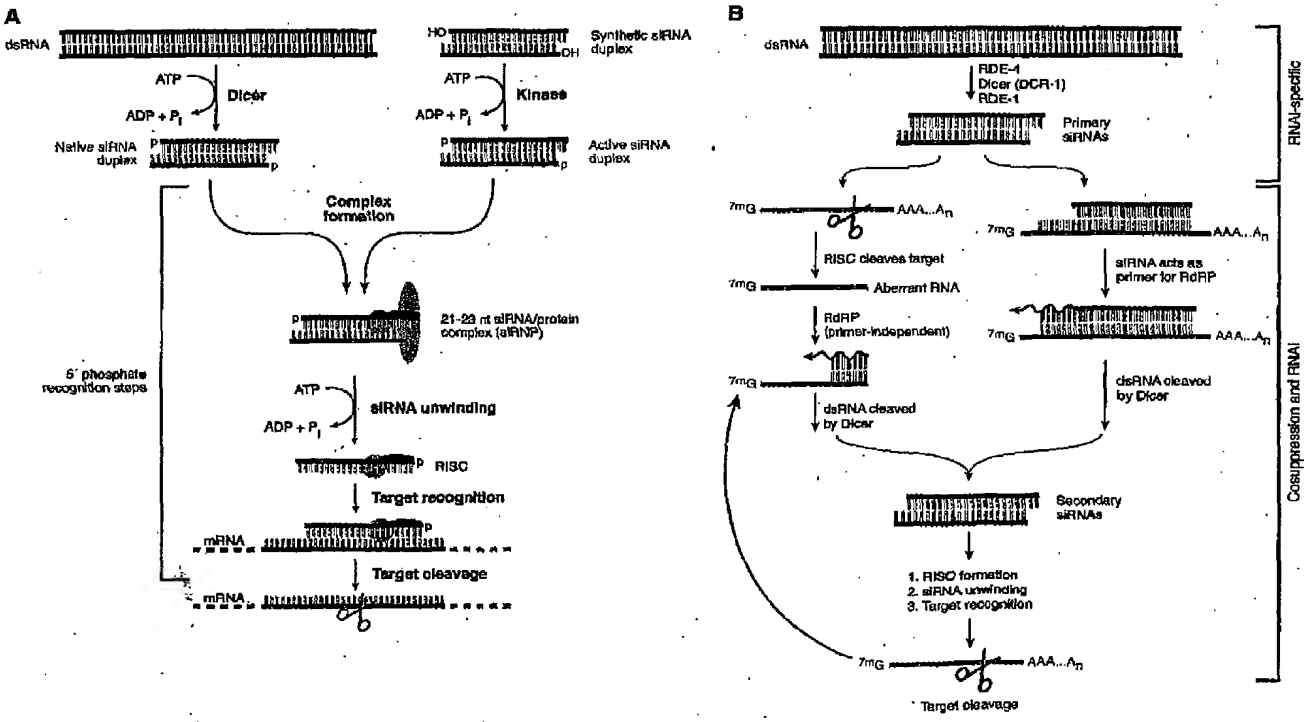

Fig. 2. Mechanisms for RNA silencing. (**A**) The "standard" model for RNAi in *Drosophila*. (**B**) A proposed but untested mechanism for RNAi and cosuppression in *C. elegans*.

they not require *rde-1* to activate the RNAi machinery? One possibility is that some other molecular abnormality triggers transposon silencing. In this view, transposon silencing might be triggered by aberrant RNA rather than double-stranded RNA. RDE-1 is also not required for cosuppression in worms (*42*, *43*).

The RDE-1 protein is a member of the PPD (PAZ and Piwi domain) family. A PPD protein is required for posttranscriptional RNA silencing in every organism where the function of this family has been tested genetically or biochemically. Another member of the PPD protein family, the RDE-1 ortholog Ago-2, is a component of the *Drosophila* RISC complex (*20*), and the PPD protein Qde-2 is associated with an siRNA-containing complex, likely a RISC, in *Neurospora* (*36*). Perhaps some PPD proteins are coupled to RdRPs that sense aberrant RNA, whereas others like RDE-1 are linked instead to proteins that bind directly to double-stranded RNA. In *C. elegans*, the RDE-1–associated protein RDE-4 is a good candidate for such a partner (*51*). Thus, RDE-4 might sense double-stranded RNA, recruit Dicer to generate primary siRNAs, then pass the primary siRNAs to downstream components of the RNAi pathway through RDE-1. Reinforcing this view, *rde-4* mutants fail to make either primary or secondary siRNAs, whereas primary siRNA levels are normal but secondary siRNAs are not made in *rde-1* mutants (*37*, *52*). In contrast, fungi mutant for the related *qde-2* gene show normal levels of siRNAs (*36*). Consistent with the idea that in worms *rde-4* is required to convert double-stranded RNA into siRNAs, whereas *rde-1* acts to shunt primary siRNAs to the cosuppression pathway, injection of short synthetic RNA duplexes partially bypasses the requirement for *rde-4*, but not *rde-1*, but only if the RNAs have the characteristic end-structure of siRNAs (*52*).

siRNAs and Other Types of Gene Silencing

In *Drosophila* and in human cells, synthetic or purified siRNA duplexes can replace double-stranded RNA as an RNAi trigger both in vitro and in vivo (*1*, *2*, *28*, *29*, *31*, *53*). Thus, siRNAs are true intermediates in the RNAi pathway in these organisms. Although siRNAs were first detected in plants, they have not yet been shown to be efficient triggers of silencing in nematodes, plants, or fungi (*52*, *54*). Is the RNAi pathway that seems to be essentially identical in flies and humans conserved more broadly? Do siRNAs serve as specificity determinants in silencing pathways other than RNAi, PTGS, and quelling? In plants, transcriptional silencing can be triggered by the introduction of transgenes that generate double-stranded RNA corresponding to the sequence of a gene's promoter. Such transcriptional silencing is accompanied by (and perhaps mediated by) methylation of the DNA sequences in the promoter region of the silenced gene (*55*, *56*). The gene is silenced because it is no longer transcribed, unlike RNAi or PTGS, in which the mRNA is transcribed at normal levels but then destroyed. Even in such promoter-based transcriptional silencing, the double-stranded RNA is converted to siRNA-like small RNAs. Determining whether these siRNAs are part of the transcriptional silencing pathway or merely reflect the nonproductive entry of a bit of the double-stranded RNA into the RNAi pathway is unknown. Support for a connection between transcriptional and posttranscriptional silencing comes from recent experiments by Birchler and colleagues, who find that the protein Piwi plays a role in the silencing of endogenous genes by homologous transgenes by both posttranscriptional and transcriptional routes (*24*).

In addition to their roles in RNAi, PTGS, and quelling, RdRPs also function in a surveillance mechanism in *Neurospora* that silences unpaired DNA at meiosis. Meiotic silencing by unpaired DNA, or "MSUD," blocks the expression of genes not found at two identical chromosomal locations during the diploid phase of the *Neurospora* life cycle (*57*). Because genes normally exist in pairs, each at the same location on sister chromosomes, unpaired genes are likely to be foreign DNA sequences, such as transposons, that pose a threat to the cell. Silencing by MSUD has been proposed to be posttranscriptional, but it is conceivable that MSUD is a form of transcriptional silencing in which specialized sensors convert the DNA sequences of unpaired genes into double-stranded RNA, which can then trigger siRNA production. Consistent with this alternative model, proteins that associate with chromatin are required for PTGS in plants (*58*), quelling in fungi (*59*), and RNAi in *C. elegans* (*60*). It is tantalizing to speculate—but harder to test—that siRNAs function both in posttranscriptional RNA silencing and in various forms of transcriptional silencing. Thus, siRNAs might not only direct the endonucleolytic destruction of a corresponding mRNA but also direct the modification of chromatin structure or the methylation of DNA, thereby turning off transcription.

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